$PGF_{2\alpha}$ inhibits VEGF Expression in the Corpus Luteum in the Mid but not Early Luteal Phase of the Sheep Estrous Cycle

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Abstract

Vascular Endothelial Growth Factor (VEGF) is important for blood vessel development in a variety of tissues including an ovarian structure, the corpus luteum (CL). The CL is a transient endocrine gland that secretes progesterone, which prepares the uterus for pregnancy. In the absence of pregnancy, the corpus luteum regresses in response to endogenous prostaglandin (PG) $F_{2\alpha}$. Deficiencies in luteal function during early pregnancy may result in embryonic loss. Understanding of the mechanisms of luteal function could aid in the development of new methods to regulate fertility.

Corpora lutea are susceptible to the luteolytic effects of $PGF_{2\alpha}$ after day 4 of the luteal phase; prior to that time the CL does not regress in response to treatment with $PGF_{2\alpha}$. The mechanisms responsible for the acquisition of responsiveness to $PGF_{2\alpha}$ and the specific intracellular mediators of $PGF_{2\alpha}$ induced luteolysis are unclear.

The objectives of this research were to investigate if exogenous $PGF_{2\alpha}$ affects VEGF mRNA expression in sheep corpora lutea and to determine if the effect is dependent upon whether the corpus luteum has not (early luteal phase CL) or has (mid-luteal phase CL) acquired luteolytic capacity.

Mature female sheep (n=18) were randomly separated into two groups (early luteal phase, day 3, and mid-luteal phase, day 10). Each group was divided into two treatment groups, $PGF_{2\alpha}$ treated and saline-treated (control). From each animal, one CL was removed at 2h and a second CL was removed 24h after treatment. Total RNA was isolated from each CL, purified, and verified for integrity. Real-time Polymerase Chain Reaction (PCR) was used for relative quantification of VEGF mRNA. The real-time methods were optimized and validated using two endogenous reference genes; β-actin and L-19. The quantity of VEGF mRNA present in each sample was standardized using

the reference genes and was expressed as fold change from the mean value for the control CL collected on day 3 at 2h. Luteal progesterone concentrations were determined using radioimmunoassay. Data were analyzed using analysis of variance for a split plot design with sheep as the main plot and CL as the sub-plot.

Quantities of VEGF mRNA were similar among CL collected during the early versus mid-luteal phase in the control animals ($p>0.05$). $PGF_{2\alpha}$ had no effect on VEGF mRNA levels in early luteal phase CL collected at 2h or 24h after injection ($p>0.05$). In contrast, on day 10 of the estrous cycle, $PGF_{2\alpha}$ treatment resulted in a marked reduction of VEGF expression within 24h after injection ($p=0.002$); it is unclear if VEGF expression was changed during the first 2h after $PGF_{2\alpha}$ administration at this stage of the cycle.

There was no effect of $PGF_{2\alpha}$ on luteal concentrations of progesterone during the early luteal phase ($p>0.05$). This is consistent with the expected lack of response at this stage. In contrast, $PGF_{2\alpha}$ caused a decrease ($p=0.03$) in luteal progesterone in mid-luteal phase CL, demonstrating that these corpora lutea have acquired luteolytic capacity.

Since the mid-cycle CL has acquired luteolytic capacity, it will regress in response to $PGF_{2\alpha}$. As such, the decline in the message for VEGF was associated with luteal regression. We conclude that inhibition of VEGF and subsequent vascular destabilization may be an important component of the luteolytic cascade invoked by $PGF_{2\alpha}$.

Introduction

Vascular Endothelial Growth Factor (VEGF) is essential for blood vessel development during fetal development, wound healing, fat deposition, and malignant tumor propagation (23). VEGF also plays an important role in ovarian function. For example, VEGF participates in the development of follicles, which are structures on the ovary that house the female gametes (25). It is also essential in the extensive vascularization of the corpus luteum (CL), a specialized ovarian gland necessary for maintenance of pregnancy (20, 21, 23).

VEGF is an angiogenic glycoprotein that binds to specific receptors on endothelial cells. VEGF consists of eight exons separated by seven introns. Five VEGF isoforms resulting from alternative splicing of the single VEGF gene have been identified in mammals. $VEGF_{189}$, $VEGF_{165}$, and $VEGF_{121}$ have been detected in the majority of cells and tissues which express the VEGF gene in humans. Various mammalian species may differ in the exact number of amino acids present in VEGF. For example, sheep VEGF has one less amino acid than the human VEGF, therefore the predominant VEGF isoforms in sheep are $VEGF_{188}$, $VEGF_{164}$, and $VEGF_{120}$ (7).

VEGF is a primary angiopoietin, helping to form primary capillary networks in previously avascular tissue during angiogenesis. Angiogenesis consists of 3 main steps: the breakdown of existing blood vessels; the migration of endothelial cells towards a stimulus; and the proliferation of endothelial cells to establish a new blood vessel sprout (9, 25, 31).

Controlled, cyclical, angiogenesis occurs naturally in the adult female in the ovary and corpus luteum. Within each estrous cycle, the CL develops from an ovulatory follicle through a process known as luteinization, which begins directly after ovulation (24). Luteinization is dependent upon angiogenesis and in order for the CL to be functional and fulfill its endocrine function, a rich vascular network must be developed (9, 25). The CL increases in size from the time of ovulation until midway through the luteal phase. This increase is due to a three fold increase in the size of the large luteal cells and a five fold increase in the number of small luteal cells (16). Seventy-five percent of the cells in a mature CL are vascular. The mature corpus luteum has one of the highest blood supplies of any organ on a per gram of tissue basis and the growth of this blood supply typically occurs within the first six days of development.

Regression of the CL is termed luteolysis and is dependent upon prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which is produced by the endometrial lining of the uterus. It has been shown that $PGF_{2\alpha}$ is a primary luteolysin for many species (1, 3, 4, 25, 30). Exogenous $PGF_{2\alpha}$ causes luteolysis in sheep when administered during the mid-luteal phase but not when administered in the first four days after ovulation (17, 24). A similar effect has been seen in cattle (28). Exogenous $PGF_{2\alpha}$ is widely used to synchronize estrus. It can also be used to induce abortions or parturition (24, 28).

Although there have been numerous studies done on the hormonal regulation of the CL, there is still much to learn about the mechanisms behind its development and regression within the ovary. Studies have been done to determine the effects of $PGF_{2\alpha}$ on a number of regulatory hormones and receptors within the ovary including progesterone, progesterone receptors, estradiol, glucocorticoids, and androgens (1, 5, 7, 27). Studies

have also looked at the changes of VEGF mRNA levels in developing female rats (8, 13), humans (5), and rhesus monkeys (10) throughout different stages of the estrous cycle. Although VEGF is an essential factor in luteal vascularization (5, 13, 22, 25) and $PGF_{2\alpha}$ induces many changes in the CL leading to luteolysis (1, 5, 7), there has been limited research done studying the effects of $PGF_{2\alpha}$ on VEGF expression during luteal formation and regression. VEGF could prove to be an important component of luteal maintenance.

The objective of the present study was to determine if $PGF_{2\alpha}$ affects the concentrations of VEGF mRNA in sheep corpora lutea. This experiment was designed to examine the acute (within 2h) vs. chronic (within 24h) effects of $PGF_{2\alpha}$ on VEGF expression in CL that have not (early luteal phase CL) or have (mid-luteal phase CL) acquired luteolytic capacity.

Materials & Methods

Experimental model and collection of CL:

Mature female sheep (n=18) obtained from the Ohio State University Sheep facility were used in this study. The sheep were randomly separated into two groups (early luteal phase, day 3, and mid-luteal phase, day 10). Each group was divided into two treatment groups, $PGF_{2\alpha}$-treated and saline-treated (control). Two CL were removed from each animal. The first one was removed at 2h and the second at 24h post treatment (Fig. 1).

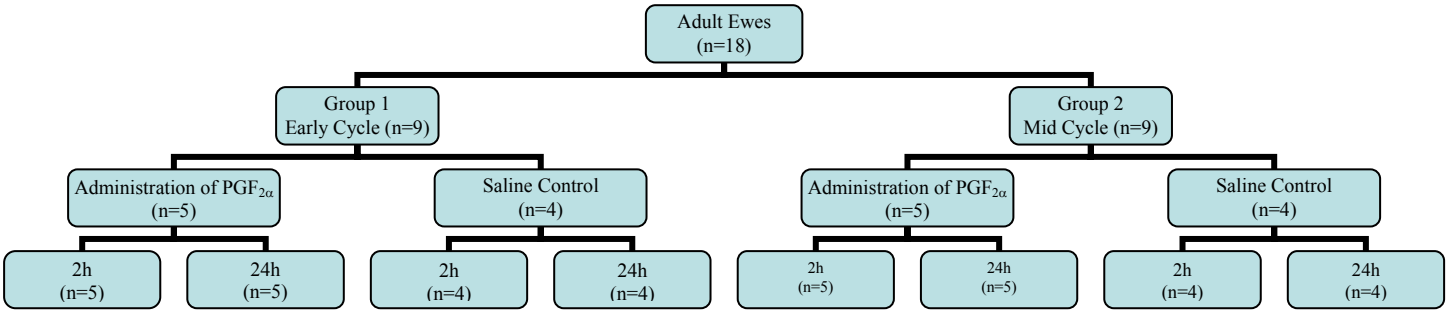

Figure 1. Experimental Design

The onset of estrus (day 0) was determined by twice daily observation in the presence of a vasectomized ram. On the assigned day of treatment (day 3 or day 10), animals were transported to the surgical facility. Ewes were sedated using pentothal. The plane of anesthesia was maintained throughout the procedure using halothane gas. A mid ventral laparotomy was performed to expose the reproductive tract. Ewes received intramuscular injections of $PGF_{2\alpha}$ (25mg dose, Lutalyse; Pharmacia & Upjohn, Kalamazoo, MI) or saline (time 0). The first CL was removed 2h post-treatment. A second CL was removed 24h post-treatment.

Luteal tissues were immediately snap frozen in liquid nitrogen. Samples were stored at -80°C. A portion of each CL was used to determine tissue progesterone concentrations by radioimmunoassay.

RNA isolation and purification:

RNA was isolated and purified from each CL using RNeasy® Mini Kit (Qiagen Inc., Valencia, CA). RNA purity and integrity was determined using the RNA NanoDrop by measuring the absorbance of each sample at 260 nm (A_{260}) and 280 nm (A_{280}). An absorbance of 1 unit at A_{260} corresponds to a 40 μg/ml RNA concentration. The total concentration of RNA in each sample was then determined according to the equation:

40 x A_{260} x dilution factor = concentration of RNA in sample (μg/ml).

The purity of the RNA sample with respect to contaminants that absorb UV light was estimated by determining the ratio of the absorbance readings at 260 nm and 280 nm (A_{260}/A_{280}) (2). Samples were also analyzed using the Bioanalyzer (Microarray Core Facility, OSU Comprehensive Cancer Center, Columbus, OH) to determine the RNA integrity. Isolated RNA was stored at -80°C.

Real Time PCR validation:

Two endogenous reference genes were selected to validate the real time PCR methods. β-actin is a structural protein present in most eukaryotic cells. β-actin has been used as an endogenous control in other reproductive studies (6, 26). L-19 is a ribosomal protein that remains fairly constant throughout the luteal phase (1).

Primers for L-19, β-actin, and $VEGF_{120}$ were used for reverse transcription of RNA from the exogenous control using SuperScript One-Step Kit (InVitrogen, Carlsbad, CA). Products were run on a 10% tris-boric acid with EDTA (TBE) gel (Bio-Rad Laboratories, Hercules, CA) and bands were visualized using UV light following staining with ethidium bromide to ensure that each set of primers amplified a product of the expected size and that only one product was being generated.

SYBR Green real-time RT-PCR was used to quantify levels of L-19, β-actin, and $VEGF_{120}$ present in each sample. SYBR Green is a method of PCR that utilizes a fluorescent molecule to quantify the amount of product present. The SYBR Green dye will only fluoresce when it is intercalated into a double stranded DNA product, however, it is not sequence specific. More double-stranded product is created with each cycle of amplification and the level of fluorescence increases proportionally. Sample concentration is measured by determining how many amplification cycles are needed to reach a threshold level of fluorescence.

It was necessary to determine an appropriate amount of RNA to include in each sample so that threshold fluorescence levels were not reached too early or late in the run; fifteen to twenty-five cycles were desirable. Each set of primers was combined with 10 fold increasing increments of exogenous control RNA and analyzed using SYBR Green real-time RT-PCR. The following amounts of sample RNA were used: 0.002ng, 0.02ng, 0.2ng, 2.0ng, 20.0ng and 200.0ng. It was determined that 20ng of sample RNA was to be used for each run. These samples were also used to generate efficiency curves to determine the efficiency of each primer. The efficiencies are used in the Pfaffl calculations (Equation 1).

Because SYBR Green is not a sequence specific method, it was necessary to determine that there were not multiple products contributing to the fluorescence level. Besides verifying this using gel electrophoresis (above), we also generated melting curves for each sample after the reverse-transcription and amplification were complete. If the product is homogeneous, a single peak will be seen.

Endogenous reference genes are critical when using the Pfaffl method to quantify RNA concentrations in real-time RT-PCR (15). The endogenous reference gene is used to correct samples for technical variation during the sample preparation. The concentrations of an effective reference gene should not vary between different samples due to time or treatment. Because endogenous reference genes are so important to the interpretation of the results for this study, both L-19 and β-actin were utilized to help ensure that we would have at least one reference gene that fit the above criteria.

A day 10, 2h, untreated animal was designated as the exogenous control. This animal was not included in the results and analysis. The RNA isolated from the animal was used to validate the RT-PCR methods. This sample was also run in each PCR sample run. This exogenous control ensured that runs were working properly and data remained consistent among different runs.

Day 3, 2h, untreated animals were chosen to be the study control (control in equation 1). The mean level of RNA present in these four corpora lutea was set as a baseline to measure either increases or decreases in $VEGF_{120}$ dependent upon time and/or treatment.

Primer Design:

β-actin forward and reverse primers were obtained from Dr. Danforth (6, 26). L-19 forward and reverse primers were obtained from Dr. Cárdenas (1). The $VEGF_{120}$ primers were designed using InVitrogen's online custom primer resources. The $VEGF_{120}$ sequence was determined by referencing work which outlined the specific exons present in each VEGF isoform (Fig. 2) (7, 13, 16). These exons were then compared to the human VEGF isoform sequences which are similar to ovine VEGF using the GenBank

available through National Institutes of Health. Sequences specific to the $VEGF_{120}$ isoform were selected so that a 152 base pair product would be generated. The reverse primer used in this study spans a segment between exons 5 and 8 (Fig. 2). $VEGF_{120}$ is the only isoform that does not have any exons between exon 5 and 8, therefore this reverse primer will only bind to the $VEGF_{120}$ isoform. Designed primers were obtained from Invitrogen™ (InVitrogen Corporation, Carlsbad, CA). The following sequences were used for $VEGF_{120}$ primers:

Forward (5' to 3') - TTATGCGGATCAAACCTCAC

Reverse (5' to 3') – TCACATTTTTCTTGTTGC

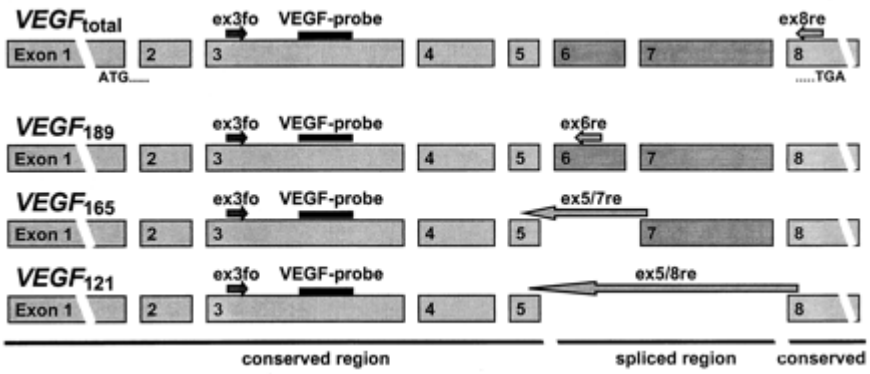

Figure 2. Human VEGF splice variants ($VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$). Figure taken from Wellman, Taube et al. (29). Figure refers to human isoforms of VEGF which differ from ovine VEGF by 1 amino acid, hence the one codon difference in all nomenclature.

VEGF Survey:

In addition to designing primers specific to $VEGF_{120}$, primers were also designed that would survey a number of VEGF isoforms including $VEGF_{120}$, $VEGF_{145}$, $VEGF_{164}$, $VEGF_{182}$, $VEGF_{184}$, and $VEGF_{205}$. This was done by referencing the previous work using

VEGF sequences and isoforms (Fig. 2) as well as GenBank (National Institutes of Health) (7, 13, 16). The designed primers were obtained from Invitrogen™ (Invitrogen Corporation, Carlsbad, CA).

Primers were combined with RNA from the exogenous control as well as from samples from each of the treatment groups. Samples were reverse-transcribed and run on a TBE gel (Bio-Rad Laboratories, Hercules, CA). Bands were visualized using UV light after staining with ethidium bromide. This allowed a quick visualization of the various VEGF isoforms present in the control and treated CL. The following sequences were used for $VEGF_{survey}$ primers:

Forward (5' to 3') - TTATGCGGATCAAACCTCAC

Reverse (5' to 3') – GGAGGCTCCTTCCTCCAG

Real-time RT PCR Analysis:

Once the system had been validated using the exogenous control, samples were run using the optimized methods (19). All samples were run in triplicate in a 96-well plate. All three sets of primers were run with each sample within the same plate. Samples from each of the four main treatment groups were selected at random and run on each plate. The exogenous control was also run in triplicate with each set of primers on each plate. QuantiTect® SYBR® Green RT-PCR Kit (Qiagen Inc., Valencia, CA) was used to prepare all samples according to manufacturer's instructions. 20ng of RNA was used in each sample well. Total reaction volume was 25µl. No transcript (NT) samples containing SYBR Green reagent and primers, but no sample RNA were also run in triplicate for each primer on each plate to ensure no contamination within the reaction reagents.

Samples were run using ABI 7900 Real-Time PCR system (ABI, Columbia, MD). Samples underwent 40 amplification cycles and the fluorescence levels were measured. Disassociation curves were generated for each sample.

Data Analysis:

The real-time PCR efficiencies (*E*) of target and reference genes were determined from the concentration curves created using the exogenous control. CT value versus the log of starting amount of RNA (ng) was plotted and the slope of the line was determined for each gene. *E* was then calculated according to the equation $E=10^{[-1/slope]}$ (15, 18, 19). The quantity of mRNA present in each sample was standardized using the reference genes according to the Pfaffl equation (Equation 1).

$$\text{Fold Change} = (E_{\text{target}})^{\Delta CT^*} / (E_{\text{reference}})^{\Delta CT^{**}}$$

Equation 1. Pfaffl Equation (15).
*$(CT\ VEGF_{control} - CT\ VEGF_{sample})$
** $(CT\ \text{Reference Gene}_{control} - CT\ \text{Reference Gene}_{sample})$

The quantity of VEGF mRNA present in each sample was expressed as fold change from the mean value for the control CL collected on day 3 at 2h (designated control). Analysis was completed using both L-19 and β-actin as reference genes.

Data were analyzed using analysis of variance for a split plot design with sheep as the main plot and CL as the sub-plot.

Luteal Progesterone Concentrations:

Luteal progesterone concentrations were determined using radioimmunoassay (RIA). These assays were completed by Ottobre and Gaddis prior to the beginning of this study. They used a proven and specific antibody (GDN 337; donated by G. Niswender,

Colorado State University) previously validated in sheep blood (11). Luteal tissue was homogenized in Tris-buffered saline. Luteal homogenates were extracted using petroleum ether prior to RIA. The progesterone assay has been validated in our laboratory for primate serum and luteal incubate (12) and porcine plasma and luteal homogenate (14). A standard curve with a slope of approximately –1.0 after log/logit transformation was first established using 2.5-500 pg progesterone standards. This assay was validated for ovine luteal homogenate by showing that when increasing volumes of homogenate (10 µl - 200 µl) were extracted and assayed for progesterone content, values were parallel to the standard curve. All samples from each animal were run in duplicate within the same assay. The inter-assay and intra-assay coefficient of variation for the progesterone assay were 12.3% and 8.4% $\pm$ 1.4%, respectively.

Results

The VEGF survey primers identified several VEGF isoforms in all samples tested. Distinct bands were visualized at 152 base pairs and 283 base pairs, which are consistent with the $VEGF_{120}$ and $VEGF_{164}$ isoforms. Another prominent band was seen at the top of the gel which may be either $VEGF_{189}$ or $VEGF_{205}$, however, the exact length of exon 6 is unknown and the exact molecular weight of these isoforms within the sheep are still unknown. In addition to these distinct bands, many light bands were seen that represent the numerous other isoforms and fragments of VEGF. No difference in concentrations between the control and treated samples could be determined by band visualization (Fig. 3).

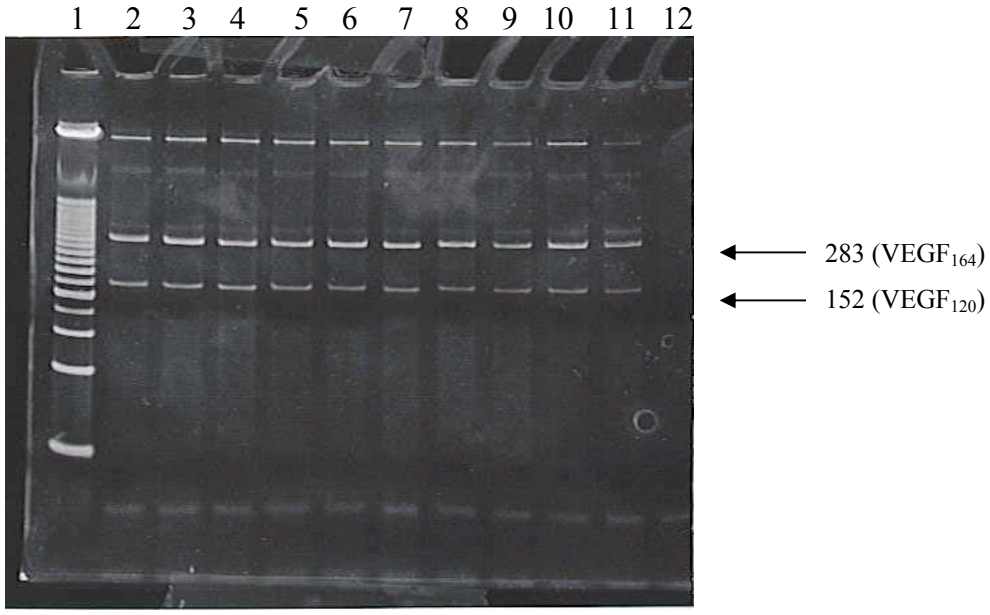

Figure 3. Visualization of VEGF Survey Primers
Samples in lanes 1-12 are listed below. All samples were amplified with the VEGF survey primers and run on a TBE Gel. Gel was visualized using UV light after staining with ethidium bromide.

Lane / Sample	Lane / Sample	Lane / Sample
1 Ladder (25 BP marker)	5 Day 3, 24h, $PGF_{2\alpha}$	9 Day 10, 24h, $PGF_{2\alpha}$
2 Day 3, 2h, Control	6 Day 10, 2h, control	10 Exogenous Control
3 Day 3, 24h, Control	7 Day 10, 24h, control	11 Exogenous Control
4 Day 3, 2h, $PGF_{2\alpha}$	8 Day 10, 2h, $PGF_{2\alpha}$	12 No Transcript

Disassociation curves displayed a single large peak for each sample (Fig. 4), which represents a homogenous product. Small disassociation peaks not consistent with sample disassociation peaks were seen in the NT samples. NT samples also increased in fluorescence at higher cycle values. This is consistent with primer dimers that can form in the absence of transcript (Fig. 5).

Quantities of RNA for both β-actin and L-19 remained constant throughout all of the samples regardless of the sample time or treatment, and individual analyses were done using each gene.

CT values for VEGF mRNA were noticeably higher 24 hours after animals were treated on day 10 (Fig. 5).

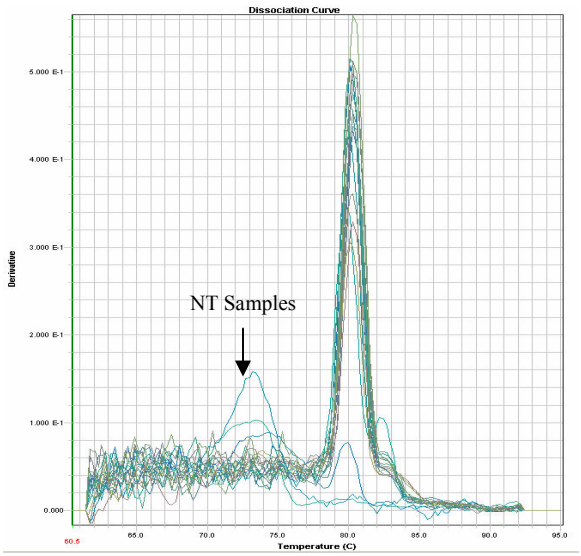

Figure 4. Dissociation curve for SYBR Green PCR run ($VEGF_{120}$).
Figure represents disassociation of $VEGF_{120}$ product run with triplicate samples from each of the four main treatment groups. Temperature is plotted on the x-axis with the derivative of the fluorescence level on the y-axis. The disassociation of each sample has a peak at the same temperature however; the NT samples can be seen to have small amounts of double stranded material present.

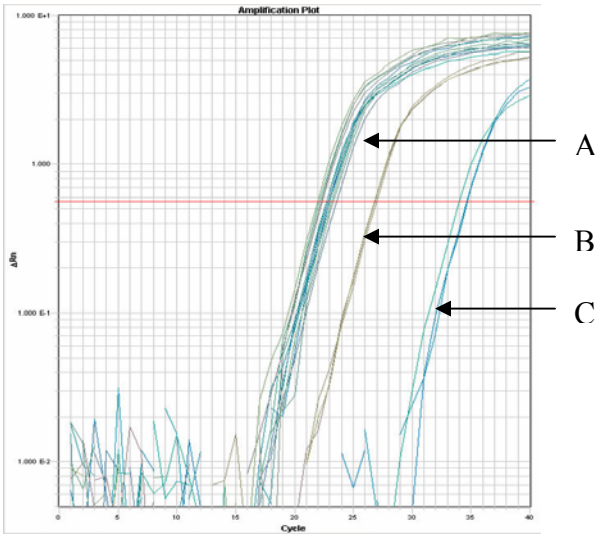

Figure 5. Amplification plot for SYBR Green PCR run ($VEGF_{120}$).
Figure represents amplification of $VEGF_{120}$ product run with triplicate samples from each of the four main treatment groups. Cycle number is plotted on the x-axis with level of fluorescence on the y-axis. The threshold fluorescence level is depicted by the red line. Samples from CL removed 24 hours after treatment for animals treated on Day 10 can be seen crossing the fluorescence threshold after approximately 25 cycles (B) while all other treatment groups cross the threshold after 20 cycles (A). The NT samples cross the fluorescence threshold after approximately 30 cycles (C).

Quantities of VEGF mRNA were similar among CL collected during the early versus mid-luteal phase in the control animals (Figs. 6 & 7, $p>0.05$). $PGF_{2\alpha}$ had no effect on VEGF mRNA levels in early luteal phase CL collected at 2h or 24h after injection ($p>0.05$). In contrast, on day 10 of the estrous cycle, $PGF_{2\alpha}$ treatment resulted in a marked (i.e., 10 fold, $p=0.002$) reduction of VEGF expression within 24h after injection. A significant increase was seen in VEGF expression 2h after $PGF_{2\alpha}$ administration when analyzed using L-19 however, this trend was not mirrored in the β-actin corrected data so it remains unclear if VEGF expression was changed during the first 2h after $PGF_{2\alpha}$ administration.

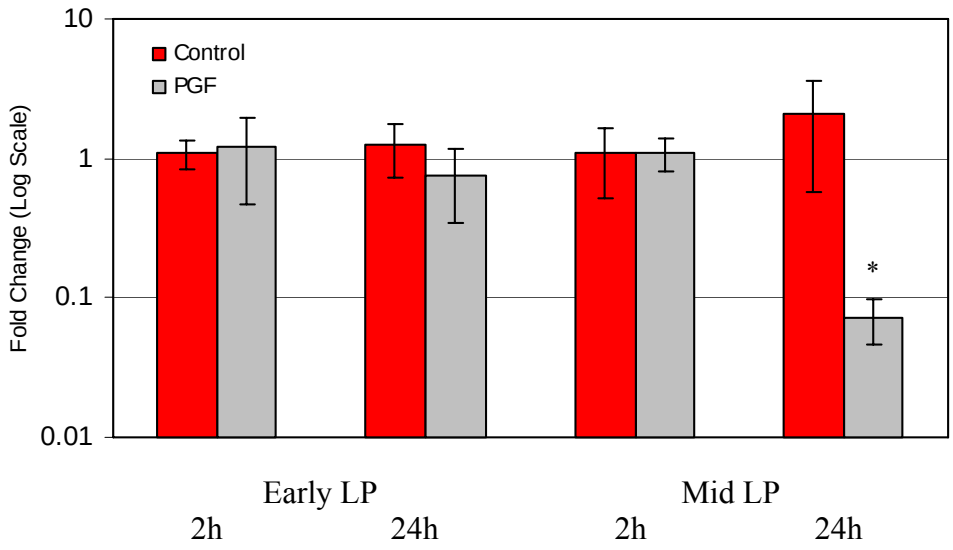

Figure 6. Fold change in VEGF mRNA in CL standardized using β-actin
Values for each study represent means +/- SE. An asterisk (*) represents significant differences between control and treated values. Data are from *n*=4 sheep for each control group and *n*=5 for each $PGF_{2\alpha}$-treated group. In each group, one CL was removed 2 hr after treatment while a second CL was removed 24 hr after treatment. Quantities of VEGF mRNA were similar among CL collected during the early versus mid-luteal phase (LP) in the control animals ($p>0.05$). On day 10 of the estrous cycle, $PGF_{2\alpha}$ treatment resulted in a marked (i.e., 10 fold, $p=0.002$) reduction of VEGF expression within 24h after injection.

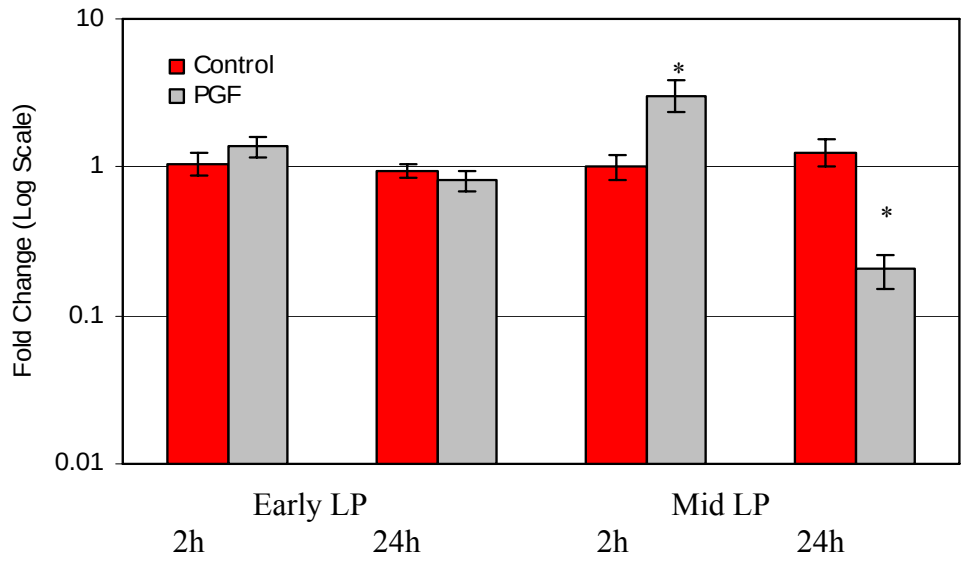

Figure 7. Fold change in VEGF mRNA in CL standardized using L-19
Values for each study represent means +/- SE. An asterisk (*) represents significant differences between control and treated values. Data are from *n*=4 sheep for each control group and *n*=5 for each $PGF_{2\alpha}$-treated group. In each group, one CL was removed 2 hr after treatment while a second CL was removed 24 hr after treatment. Quantities of VEGF mRNA were similar among CL collected during the early versus mid-luteal phase (LP) in the control animals ($p>0.05$). On day 10 of the estrous cycle, $PGF_{2\alpha}$ treatment resulted in a small but significant increase ($p=0.002$) in VEGF expression 2 hours after injection. On day 10 of the estrous cycle, $PGF_{2\alpha}$ treatment resulted in a marked (i.e., 5 fold, $p=0.002$) reduction of VEGF expression within 24h after injection.

There was no effect of $PGF_{2\alpha}$ on luteal concentrations of progesterone during the early luteal phase (Fig. 8, $p>0.05$). This is consistent with the expected lack of response at this stage. In contrast, $PGF_{2\alpha}$ caused a decrease ($p=0.03$) in luteal progesterone in mid-luteal phase CL (both 2h and 24h after $PGF_{2\alpha}$ administration), demonstrating that these corpora lutea have acquired luteolytic capacity.

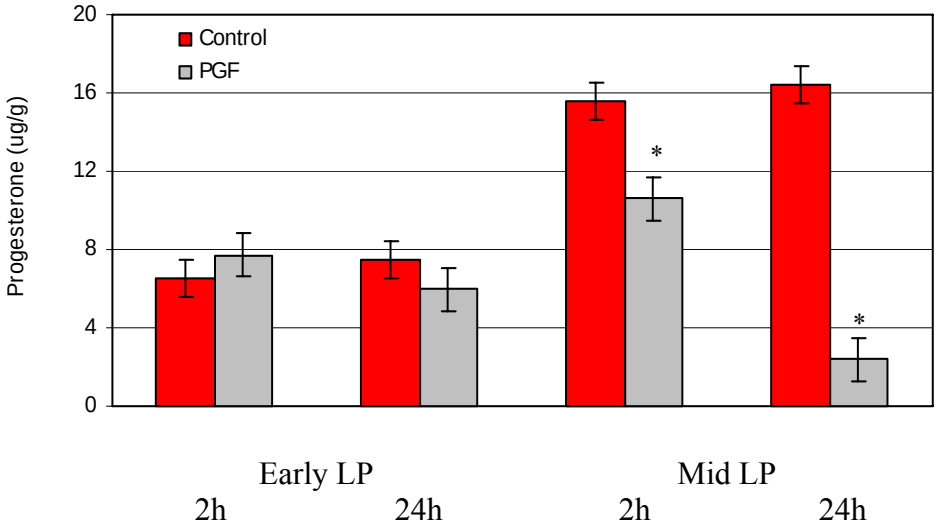

Figure 8. Progesterone concentrations in corpora lutea
Values for each study represent means +/- SE. An asterisk (*) represents significant differences between control and treated values. Data are from *n*=4 sheep for each control group and *n*=5 for each $PGF_{2\alpha}$-treated group. In each group, one CL was removed 2 hr after treatment while a second CL was removed 24 hr after treatment. There was no effect of $PGF_{2\alpha}$ on luteal concentrations of progesterone during the early luteal phase (LP) ($p>0.05$). This is consistent with the expected lack of response at this stage. In contrast, $PGF_{2\alpha}$ caused a decrease ($p=0.03$) in luteal progesterone in mid-luteal phase CL (both 2h and 24h after $PGF_{2\alpha}$ administration).

Discussion

$PGF_{2\alpha}$ causes a significant decrease in VEGF mRNA concentrations 24 hours after treatment in mid luteal phase CL, but not in early luteal phase CL. This coincides with the acquisition of luteolytic capacity, as such capacity is present by day 10 of the luteal phase.

The response of $VEGF_{120}$ can be compared to that of progesterone. Neither show any marked decrease in the early luteal phase CL but do show a significant decrease in concentration in the mid luteal phase CL. These two products both are affected by the ability of the CL to regress. Progesterone is an endocrine product secreted by luteal cells

in a functional corpus luteum. Therefore, it makes sense that there is an acute, sustained decrease in concentration as the CL begins to undergo regression. $VEGF_{120}$ plays a structural role within the CL and is locally acting rather than an endocrine product.

Another interesting comparison can be made between the VEGF data and data acquired from Ottobre and Gaddis on work done involving changes in Vitamin C concentrations throughout the luteal phase due to treatment with exogenous $PGF_{2\alpha}$. Ottobre and Gaddis measured the change in vitamin C concentrations in relation to the day of the cycle and to the treatment with $PGF_{2\alpha}$. Treatment with $PGF_{2\alpha}$ causes a decrease in vitamin C concentrations both in the early and mid luteal phase. However, in the early luteal phase, vitamin C concentrations are decreased at 2 hours post treatment but have returned to normal levels by 24 hours post treatment. An overall increase in vitamin C concentrations between day 3 and day 10 is also observed.

These data are of great interest, because they show that the early luteal phase CL is responsive to $PGF_{2\alpha}$ treatment, even though the CL has not acquired lutealytic capacity. This helps us to understand that $PGF_{2\alpha}$ has pathway specific effects throughout the entire luteal phase.

The decrease in $VEGF_{120}$ mRNA concentrations in mid but not early luteal phase CL should not be surprising. VEGF is a vascularizing protein and the early luteal phase CL is an intact gland that requires a high degree of vascularization to function. However, as a CL undergoes regression there is no need for vascular proliferation. The delayed effect of $PGF_{2\alpha}$ on the concentrations of $VEGF_{120}$ reinforces the belief that VEGF plays a structural role within the CL, rather than a functional role. Though the function of the CL

may be affected soon after treatment of $PGF_{2\alpha}$, the structural changes that occur with regression take longer to materialize.

Since the mid-cycle CL has acquired luteolytic capacity, it will regress in response to $PGF_{2\alpha}$. As such, the decline in the message for VEGF was associated with luteal regression. We conclude that inhibition of VEGF and subsequent vascular destabilization may be an important component of the luteolytic cascade invoked by $PGF_{2\alpha}$. It is currently still unclear whether $PGF_{2\alpha}$ has a direct effect on VEGF concentrations or if the effects are an indirect result of the luteolytic process. Future studies might look directly at the effects of $PGF_{2\alpha}$ on VEGF concentration present in luteal tissue rather than studying concentrations of mRNA.

A better understanding of the mechanisms behind luteal development and regulation could aid in the development of new methods to increase fertility and synchronize estrus. Alternatively, by controlling luteal regression, one would have the means for compromising fertility. The functional corpus luteum inhibits ovulation and is essential for maintaining pregnancy. In the absence of pregnancy, the corpus luteum regresses in response to $PGF_{2\alpha}$. The loss of the corpus luteum during early pregnancy leads to miscarriages and spontaneous abortions (7, 24, 30).

This is of great interest to the livestock industry. In the beef industry, heifer cattle grow faster and have a much higher feed efficiency if they are not pregnant. If the corpus luteum could be maintained in the absence of pregnancy, ovulation and therefore pregnancies would be prevented. In contrast, a better understanding of how the corpus luteum functions to maintain pregnancy could lead to a decrease in the number of spontaneous abortions within herds.

This research is not only beneficial to the livestock industry, but the human health fields as well. As women are waiting longer to have children, fertility treatments and alternatives are becoming increasingly important. Corpora lutea regulation has similarities across many mammalian species. The methods of enhancing or interrupting fertility may be applicable to a human model.

VEGF plays a role in many fields outside of reproduction. Vascularization is involved in wound healing and tumor proliferation. The complex mechanisms of development and rapid growth of the CL are very similar to mechanisms involved in tumor growth and wound healing (22, 23). Tissues within the adult female reproductive system, much like tissues of tumors, proliferate at extremely high rates as compared to endothelial cells. The ovarian corpus luteum is able to double in size and cell number every sixty to seventy hours (22). The ovary is the only site where rapid cell proliferation occurs in a highly controlled, cyclical state. These attributes make the corpus luteum an excellent model for studying tumor proliferation. A better understanding of the regulation of angiogenesis (blood vessel development) and VEGF expression within the ovary can lead to new treatments for tumors and wounds.

The ability to regulate VEGF expression within the body would be a powerful medical tool in both animal and human medicine.

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